



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

A tripod anchor offers improved robustness of peptide-based electrochemical biosensors

Citation for published version:

Staderini, M, González-fernández, E, Murray, AF, Mount, AR & Bradley, M 2018, 'A tripod anchor offers improved robustness of peptide-based electrochemical biosensors', *Sensors and Actuators B: Chemical*.
<https://doi.org/10.1016/j.snb.2018.07.100>

Digital Object Identifier (DOI):

[10.1016/j.snb.2018.07.100](https://doi.org/10.1016/j.snb.2018.07.100)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Version created as part of publication process; publisher's layout; not normally made publicly available

Published In:

Sensors and Actuators B: Chemical

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Accepted Manuscript

Title: A tripod anchor offers improved robustness of peptide-based electrochemical biosensors

Authors: Matteo Staderini, Eva González-Fernández, Alan F. Murray, Andrew R. Mount, Mark Bradley



PII: S0925-4005(18)31348-0
DOI: <https://doi.org/10.1016/j.snb.2018.07.100>
Reference: SNB 25069

To appear in: *Sensors and Actuators B*

Received date: 16-3-2018
Revised date: 19-7-2018
Accepted date: 22-7-2018

Please cite this article as: Staderini M, González-Fernández E, Murray AF, Mount AR, Bradley M, A tripod anchor offers improved robustness of peptide-based electrochemical biosensors, *Sensors and amp; Actuators: B. Chemical* (2018), <https://doi.org/10.1016/j.snb.2018.07.100>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

A tripod anchor offers improved robustness of peptide-based electrochemical biosensors

Matteo Staderini,^{a§} Eva González-Fernández,^{a§} Alan F. Murray,^b Andrew R. Mount,^{a*} Mark Bradley^{a*}

^a EaStCHEM, School of Chemistry, University of Edinburgh, Joseph Black Building, West Mains Road, Edinburgh, EH9 3FJ, UK.

^b School of Engineering, Institute for Bioengineering, The University of Edinburgh, The King's Buildings, Mayfield Road, Edinburgh EH9 3JL, UK.

[§]These authors contributed equally to this study

*Corresponding authors. Fax: 0044 131 777 0334

E-mails: mark.bradley@ed.ac.uk (M. Bradley); a.mount@ed.ac.uk (A.R. Mount)

Highlights

- A methylene blue-tagged peptide-based probe endowed with a triple anchor was generated for the first time.
- The tripod anchor probe was used to produced SAMs with enhanced long-term, thermal and chemical stability
- The tripod anchor probe showed efficient electron transfer and protease detection.

Abstract

Peptide-based electrochemical biosensors have proven to be powerful sensing strategies for the detection of proteases and offer a highly versatile system as simple tuning of the immobilised substrate peptide leads to a new sensing platform. The most common immobilisation strategy of peptides onto an electrode surface is via a self-assembled monolayer (SAM) through a thiol group that can be readily chemically incorporated in the

target peptide. However, the successful application of these platforms in complex biological samples can be frustrated by the lack of stability of the peptide-based SAM, that can lead to false positives and limited shelf-life. Herein, we investigated the stability of a peptide-based electrochemical platform endowed with a single or a tribranched thiol group for attaching onto the electrode surface. Side-by-side comparison demonstrated that the tripod anchor generated a highly robust peptide-based electrochemical biosensor that showed improved stability when compared to the monoanchor analogue, simplifying data analysis and interpretation, while showing efficient protease detection and similar sensing capabilities.

Keywords Peptide-based probe SAMs stability Protease detection Tripod anchor

1. Introduction

Self-assembled monolayers (SAMs) have been used for the fabrication of a vast range of devices encompassing organic electronics [1], molecular motors [2], sensors and actuators [3]. However, it is in the field of electrochemical biosensors where SAMs have found the most general practical application by providing an effective and versatile system for functionalising electrode surfaces [4, 5]. Among the different types of SAMs, thiols on gold, which typically comprise a sulfhydryl anchoring group attached to a spacer followed by a terminal cargo such as a specific strand of DNA, an aptamer or peptide, have received much attention. In part, this is due to their ease of preparation, driven by the readily formed bond between the sulfhydryl group and the gold surface [6, 7].

However the Au-S bond, which has a semi-covalent character (with a bond strength of the order of 50 kcal/mol) [7] is quite labile and alkanethiol-based SAMs show significant instability issues which limit their practical use in sensing. The singly bound sulfhydryl groups can be readily displaced from the surface by other thiols, while prolonged storage in phosphate buffer

[8, 9], exposure to elevated temperatures [10] and high salt concentrations [8] can result in desorption of a substantial proportion of the thiolates within a few days [11].

In order to improve film stability, a variety of anchoring strategies have been explored [12, 13]. One attractive approach relies on employing multidentate adsorbates; the increased number of thiols are then able to form multiple bonds with the surface enhancing the robustness of the monolayer [14-16]. Indeed, trithiol based-anchors have proven to be suitable for robust bioconjugation onto gold surfaces and have been employed for attaching DNA strands onto gold thin-films [17] and nanoparticles [18-20]. As regards electrochemical biosensors, SAMs of dithiolated scaffolds have been exploited for the attachment of antibodies [21] and oligonucleotides [22], while a trithiol anchor has been used to produce a highly stable DNA sensor platform [23, 24]. However, the tridentate (tripod) anchor approach has not been investigated in the preparation of SAMs in peptide-based electrochemical biosensors. Peptides have been immobilised on electrode surfaces through Au-S bonds and used for the detection of metastatic activity [25], proteases [26], cortactin [27] and other type of proteins [28]. In this context, SAMs need to provide: (1) the correct orientation of immobilised peptides to enable analyte detection, (2) an appropriately packed structure that allows both electron transfer and protein detection and (3) enhanced stability [29].

In our recent work, we reported an electrochemical peptide-based biosensor for trypsin which consisted of a short peptide sequence, labelled with methylene blue (MB) as a redox reporter and immobilised onto a gold electrode surface using cysteine as the thiol-containing anchor [30].

In an effort to improve the robustness of this biosensor, we herein describe the synthesis of a tripod anchor probe and its application for the generation of more robust SAMs. We report on the sensing abilities of the new electrochemical biosensor, its stability under a range of conditions, while comparing its analytical performance to that of the benchmark monodentate probe as a control.

2. Materials and methods.

2.1. Instrumentation

Electrochemical measurements were performed using a conventional three-electrode electrochemical cell driven by a computer-controlled AutoLab PGstat-30 potentiostat running the GPES 4.9 software (EcoChemie, The Netherlands). A platinum wire and a 2 mm diameter polycrystalline gold electrode (IJ Cambria, UK) were used as auxiliary and working electrode, respectively. All the potentials are referred to the Ag|AgCl|KCl (3 M) reference electrode (Bioanalytical Systems, Inc., USA). ^1H and ^{13}C nuclear magnetic resonance spectra were recorded on a Bruker AVA500 spectrometer (500 and 126 MHz respectively) at 298 K in deuterated solvents. Chemical shifts are reported on the δ scale in ppm and for ^1H are referenced to residual non-deuterated solvent resonances. Infrared absorption spectra were recorded on a SHIMADZU. Irtaffinity-1 CE FTIR spectrometer. Normal phase purifications by column chromatography were carried out on silica gel 60 (230-400 mesh). Analytical reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on an Agilent 1100 system equipped with a Discovery C18 reverse-phase column (5 cm x 4.6 mm, 5 μm) with a flow rate of 1 mL/min and eluting with $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{HCOOH}$ (95/5/0.05) to $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{HCOOH}$ (5/95/0.05), over 6 min, holding at 95% CH_3CN for 3 min, with detection at 650 nm and by evaporative light scattering. High Resolution Mass Spectra (HRMS) were performed on a Bruker 3.0 T Apex II spectrometer. MALDI TOF MS were run on a Bruker Ultraflextreme MALDI TOF/TOF with a matrix solution of sinapic acid (10 mg/mL) in $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{TFA}$ (50/50/0.1).

2.2. Reagents

Trypsin (MW 23.4 KDa), bovine serum albumin (BSA), 6-mercaptohexanol (MCH), 2,2'-(ethylenedioxy)diethanethiol (PDT) and 10x PBS were purchased from Sigma Aldrich (UK) and used as received. Amino acids and the polystyrene resin with a 2-chlorotriylchloride linker were purchased from GL Biochem (Shanghai) Ltd and NovaBiochem, and all other chemicals from Sigma Aldrich and Acros. All reagents were of analytical grade. All solutions were prepared using protease-free deionised water.

2.3. Experimental methods

2.3.1. Synthesis

The detailed synthetic experimental procedures are described in Appendix A: Supplementary Data.

2.3.1. Electrode cleaning and pre-treatment

After immersing in the minimum volume of piranha solution (3:1-H₂SO₄ (95%): H₂O₂ (33%)) (CAUTION: piranha solution is strongly oxidising and should be handled with care!) for 10 min in order to eliminate any organic matter from the gold surface, the working electrode was successively polished on a polishing cloth using alumina slurries of 1, 0.3 and 0.05 µm particle size (Buehler, Germany). This working electrode was then further cleaned by immersion in H₂SO₄ (95%) and then HNO₃ (65%) at room temperature for 10 min. Finally, the working electrode was subjected to cyclic voltammetry, carrying out potential cycles between 0 and +1.6 V in 0.1 M H₂SO₄ at a scan rate of 100 mV·s⁻¹ until the characteristic voltammogram of clean polycrystalline gold was obtained.

2.3.2. Sensing phase preparation protocol

The sensing phase was formed as a mixed SAM on the gold electrode surface by immersing the freshly cleaned and pre-treated gold working electrode overnight at 4 °C in a ethanolic solution of the redox-labelled peptide (40 µM) and freshly prepared PDT (600 µM). After washing with ethanol, the resulting SAM-modified electrode was immersed in 1 mM MCH in ethanol for 1 hour at room temperature. Finally, two washing steps were carried out, first in ethanol and then in phosphate-buffered saline (PBS). The modified electrodes were then stored in PBS solution at 4 °C until use.

2.3.3. Sensor measurements

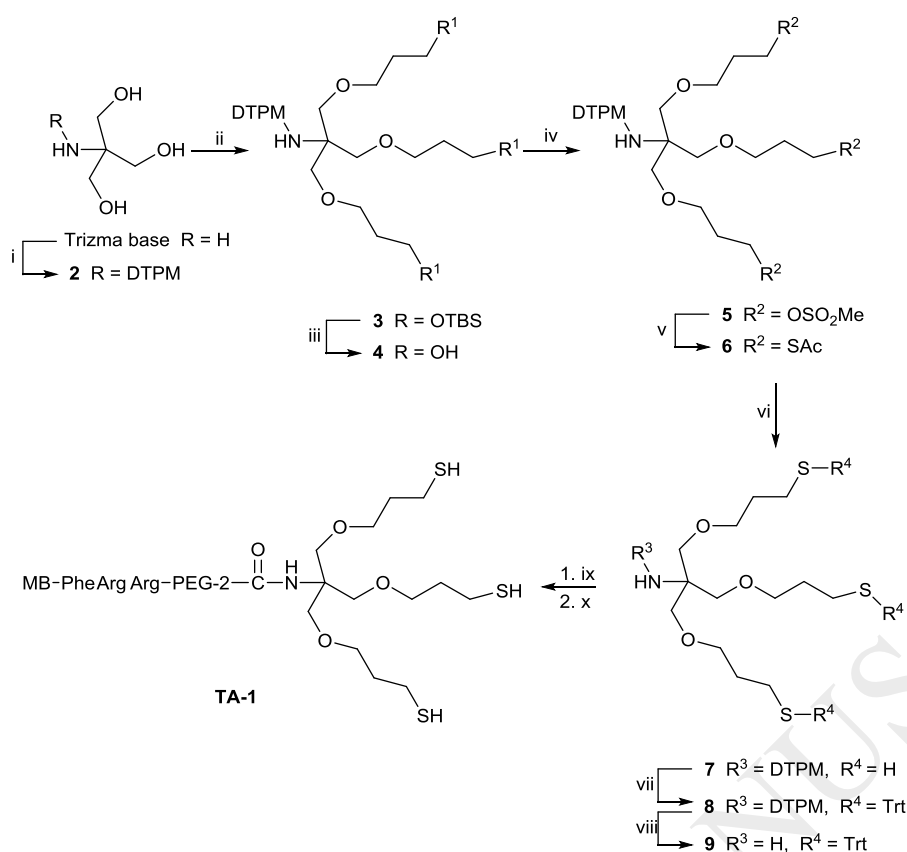
The modified electrodes were immersed in 1x PBS buffer solution (pH 7.4, optimum in terms of trypsin catalytic activity) and electrochemically interrogated using square wave voltammetry

(SWV, at a frequency of 60 Hz, amplitude of 25 mV and step potential of 5 mV) until a stable background signal was obtained. After addition of the target enzyme, the SWV signal was continuously monitored with time, with the resulting signal gain being expressed as the relative change in SWV peak current with respect to the initial peak current (henceforth called the % signal change).

3. Results and discussion

3.1. Synthesis of the tripod and monoanchor probes

Figure 1A shows the tripod and the monoanchor peptide-based probes (TA-1 and MA-1). TA-1 was synthesised (Scheme 1) through protecting trizma base with DTPM (**2**) [31], followed by the addition of (3-bromopropoxy)-tert-butyldimethylsilane in the presence of NaH and the removal of the TBS protecting groups to give **4**. The resulting hydroxyl groups were transformed into methylsulfonates which were displaced using potassium thioacetate to give **6**. Hydrolysis (HCl/MeOH), then reprotection of the thiols with trityl groups, and amino group deprotection with hydrazine gave the trialkylthiol anchor **9**. The redox labelled peptide **10** (MB-Phe-Arg-Arg-PEG-2-OH) was synthesised using Fmoc solid-phase chemistry on a polystyrene resin with a 2-chlorotrytyl chloride linker as described in the Appendix A (Scheme S2). This redox peptide was then coupled with the trialkylthiol anchor **9** to give the desired probe TA-1. Synthesis of the corresponding MA probe followed the same protocol but starting from ethanolamine (Appendix A, Scheme S1).



Scheme 1. Synthesis of TA-1. Reaction conditions: i) DTPM-NMe₂ 1, MeOH, overnight; ii) (3-bromopropoxy)-tert-butyldimethylsilane, NaH, DMF, 24 h; iii) TBAF, THF, overnight; iv) MeSO₂Cl, Et₃N, DCM, 3 h; v) KSAc, anhydrous THF, overnight; vi) HCl/MeOH (1/4), 5 h, reflux; vii) triphenylmethyl chloride, DCM, 3 h; viii) hydrazine monohydrate, 1 h; ix) MB-Phe-Arg-Arg-PEG-2-COOH 10, HOBt, EDC, DMF, 24 h x) TFA/EDT/water/TIS (94/2.5/2.5/1), 30 min.

3.2 Characterisation of tripod- and monoanchor-peptide SAMs

To investigate the ability of both MA-1 and TA-1 to produce an active sensing layer able to support electrochemical protease detection, SAMs were prepared following a previously described protocol, which involves ternary SAM formation using a combination of probe, ethylene glycol-based dithiol and mercaptohexanol, previously optimised for generating a monodentate-based SAM for protease detection [30]. The successful formation of both redox

active SAMs on the gold surface was confirmed by cyclic voltammetry (CV), which showed a linear dependency of the measured MB peak current on the scan rate (in the range 10-200 mV/s), confirming that the electrochemical response is due to surface-bound redox species (Figure S1). Both TA and MA modified surfaces (referred to as the TA and the MA-sensor) were then challenged with trypsin. The initial current values measured by square wave voltammetry (SWV) were -1.9 ± 0.5 (n=19) and -6 ± 2 μA (n=14) for the MA and TA-sensors respectively. This is consistent with a higher amount of TA probe on the surface due to its increased binding energy relative to the backfilling molecules (ethylene glycol-based dithiol and mercaptohexanol) arising from multiple thiol-Au anchoring. Continuous electrochemical interrogation of both sensing layers by SWV showed the expected decrease of the registered current intensity with time in each case, due to trypsin catalysed cleavage and the release of the redox-containing fragment into solution (Figure 1B). It is interesting that the percentage change in peak current after 90 min was the same within experimental error, $56 \pm 8\%$ and $58 \pm 4\%$, for MA-1 and TA-1 layers, respectively. Additionally, the percentage signal change for TA-1 also followed the Michaelis-Menten kinetics model previously established for MA-1, which describes the heterogeneous enzymatic cleavage of the methylene blue-tagged peptides (Figure 1B, insert) [30]. Fitting to this previously proposed model allowed extraction of the effective cleavage rate constant (k_{eff}) values of 0.077 ± 0.005 and 0.038 ± 0.002 min^{-1} respectively for MA-1 and TA-1, through relating the fractional cleavage of the immobilised MB-tagged probe (θ) with time through the equation: $\theta = 1 - e^{-k_{\text{eff}}t}$ (Eq. 1). This suggests that the MA-1 probe film structure and density results in slightly faster enzymatic cleavage than the TA-1. However, this did not result in a significant difference in overall signal change within the 90 min observation period. That the % signal change did not reach 100% again indicates the presence of non-accessible/non-cleavable probes, previously attributed to be a consequence of the polycrystalline gold roughness [32].

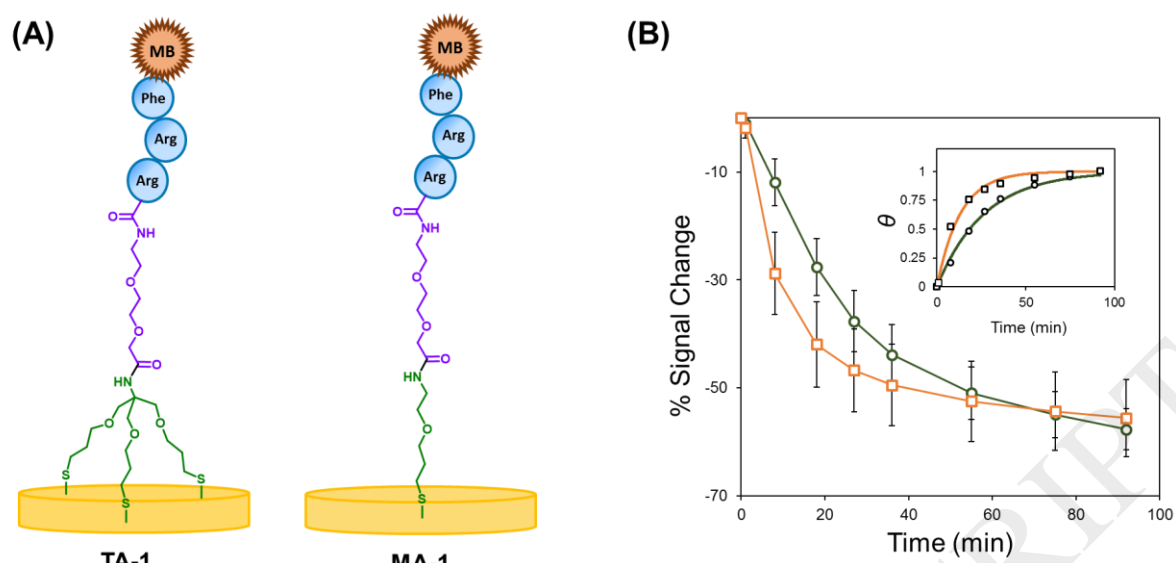


Figure 1. (A) Representation of the triple and monoanchor probes (TA-1 and MA-1) immobilised onto a gold surface electrode. (B) Percentage MB peak current change with time upon the addition of trypsin for the TA-1 (green circles) and MA-1 surface layers (orange squares). Insets show the calculated fraction of cleavage, θ , vs time plot, and best fit to equation (1) [30]. All points and error bars represent the average and standard deviation respectively of the response from 3 individual sensing layers.

The electron transfer (ET) kinetics exhibited by both probe films were compared using SWV data recorded at varying frequencies and the Komorsky-Lovrić-Lovrić formalism for surface-tethered redox species [33, 34]. Plotting the (I/f) relation vs $(1/f)$ (where f is the interrogating frequency, and I the measured redox peak height) showed a maximum, or critical frequency, f_c , which is related to the ET rate (k_s) through $k_s = \omega_{\max} f_c$ (Eq. 2), with ω_{\max} being a theoretically calculated kinetic parameter (1.18 in this particular case) [33]. The resulting values of maximum frequency corresponded to a k_s of 70.8 s^{-1} for the MA-1 probe (Figure 2A) and 82.6 s^{-1} for the TA-1 probe (Figure 2B). From these data, it can be concluded that both probes support efficient electron transfer with comparable ET rates in the buffer solution used throughout this work, PBS 1x (pH 7.4). The observed k_s values are also comparable to those previously reported for equivalent systems [23, 35].

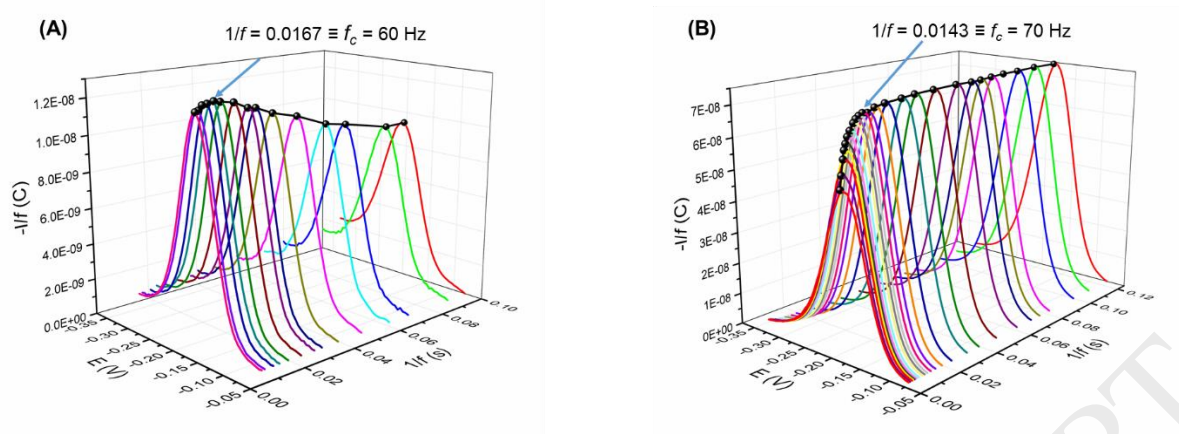


Figure 2. SWV normalised to the corresponding frequency, f , at which they were recorded versus $1/f$ in PBS for (A) MA-sensor and (B) TA-sensor using an amplitude of 25 mV and a step potential of 5 mV.

3.3. Analytical performance comparison for tripod- and monoanchor-peptide SAMs as electrochemical platform for trypsin detection.

In order to evaluate the analytical characteristics of both probes, MA-1 and TA-1 modified surfaces were immersed in solutions containing varying concentrations of trypsin (1-100 nM) in PBS. The electrochemical signal measured by SWV was monitored in real-time and the peak current expressed as % signal change for 90 min. As expected, the higher the enzyme concentration in solution, the faster the % signal change that was registered - in line with faster proteolytic cleavage of the methylene blue-tagged peptide immobilised on the gold electrode (Figure 3). Plotting the % signal change at 90 min, taken as a practical maximum timescale of detection, against the concentration of trypsin showed a linear relationship, allowing both sensing platforms to be evaluated and compared (Figure S2). It is worth noting that the signal can be taken at shorter times, and thus, allowing tuning of the time-to-signal to the required sensing concentration range depending on the specific requirements for each application, with potentially faster detection. The analytical characteristics are given in Table 1. The limits of detection (LOD), calculated as the signal corresponding to the blank minus 3 times the standard deviation for the blank and the dynamic ranges were of the same order of magnitude,

confirming the ability of the tripod anchor probe to provide a suitable sensing platform for trypsin. Furthermore, the tripod probe signal was shown to be sufficiently stable to preclude the need to measure and subtract a negative control response, thereby simplifying analysis at low analyte concentration.

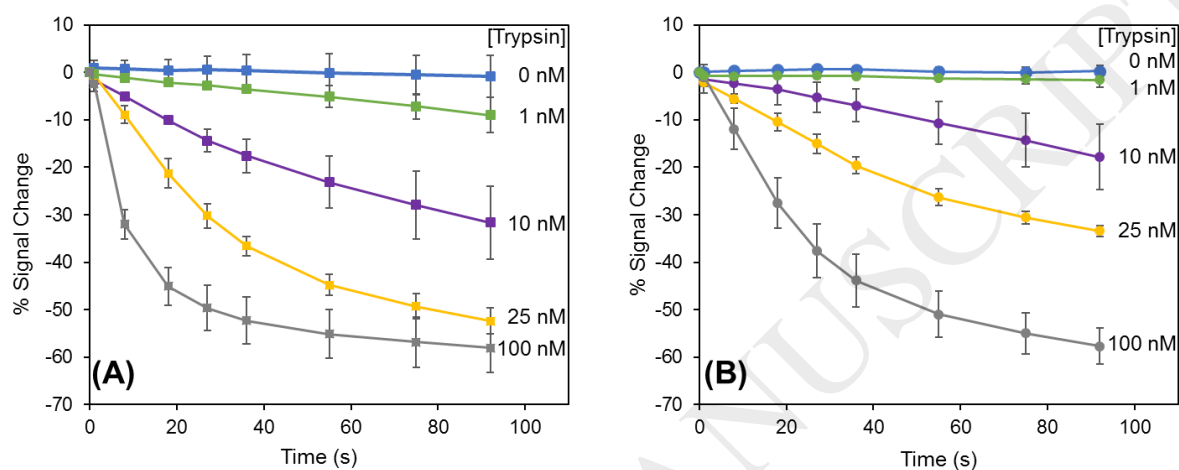


Figure 3. Percentage MB peak current change with time upon the addition of varying trypsin concentrations (0, 1, 10, 25 and 100 nM) for (A) MA-1 and (B) TA-1 modified surfaces.

Table 1. Analytical characteristics of MA-1 and TA-1 sensors for trypsin detection.

Sensor	LOD (nM)	Measured range (nM)	CV%	for	CV%	for
			[trypsin]=100 nM		[trypsin]=25 nM	
MA-1	2.3	1-100	9		5.3	
TA-1	1.2	1-100	6.6		3.3	

CV%: coefficient of variation.

3.4. Stability evaluation for tripod- and monoanchor-peptide SAMs

The stability of the TA-1 probe film sensors was assessed in terms of thermal and chemical stability and compared to the analogous monoanchor MA-1 system. Stability at 4 °C in PBS

was evaluated periodically over 30 days by measuring the SWV signal. As shown in Figure 4A, the TA-1 system exhibited enhanced storage stability when compared to the MA-1 system. This was observed both in the first 15 days, when TA-1 underwent around half the signal loss of MA-1, and in an essentially unaltered response subsequently up to 30 days, in contrast to MA-1. Immersion in PBS at 40 °C for 2 h and periodically measuring the SWV signal (Figure 4B) also showed enhanced TA-1 stability, with the MA-1 signal decrease of 45% at 2 h, contrasting with the 20% registered for TA-1. Both TA and MA-sensors when challenged with PBS containing 2 mM DTT showed that the TA-sensor was totally stable, whereas there was continuous drop in signal for the MA-sensor (Figure 4C). This suggests that TA confers SAM-based sensors enhanced stability against competing reducing thiols that can be present in real samples, such as glutathione, or cysteine-rich proteins.

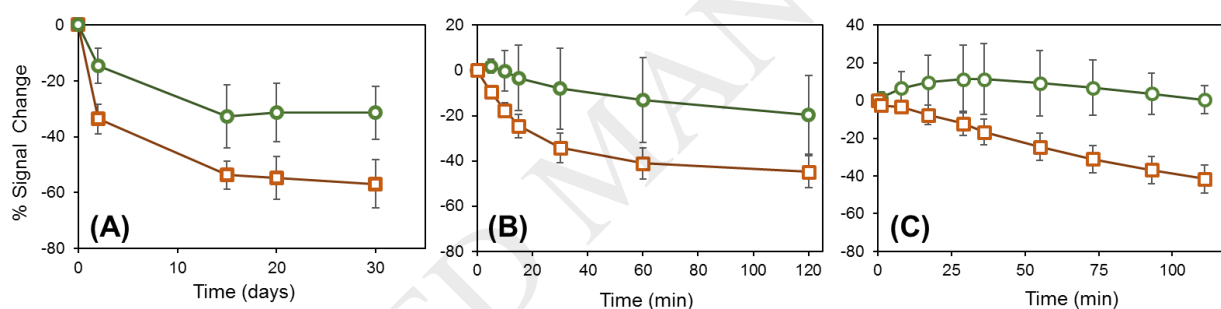


Figure 4. Percentage signal change registered for TA-1 (green circles) and MA-1 (orange squares) probe films when (A) stored in PBS at 4 °C for 30 days; (B) immersed in PBS at 40 °C for 2 h; and (C) immersed in PBS containing 2 mM DTT at room temperature.

Overall TA-based sensors show enhanced stability under all tested conditions, arising from the enhanced affinity that the triple anchor has once bound to the gold surface. Furthermore, tripod-modified surfaces rendered appropriate outputs upon enzyme exposure after each of the stability conditions tested in terms of absolute value and reproducibility (Figure S3). This outcome is in-line with previously reported multi-anchoring systems such as used for DNA-based electrochemical sensors [23], optical biosensors [17] or nanoparticle decoration [36],

although this is the first time this has been applied to peptide-based electrochemical biosensors.

4. Conclusions

In summary, a methylene blue-tagged peptide-based probe endowed with a triple anchor was generated, immobilised onto a gold electrode surface, and employed for the detection of protease activity. The resulting SAMs displayed improved long-term, thermal and chemical stability compared to the more traditional single anchor strategies, resulting in a more stable background signal with time and comparable analytical performance when compared to their monoanchor analogue. This results in a lower limit of detection and also voids the requirement for the measurement and subtraction of the background (negative control) response. The broader application of the proposed triple anchor will provide a versatile tool for the development of robust electrochemical sensors for various bio-sensing applications and their use in real complex samples, particularly where they have to be stored for a significant period of time between their manufacturing and their usage dates.

Acknowledgements

The authors acknowledge financial support from the EPSRC-funded Implantable Microsystems for Personalised Anti-Cancer Therapy (IMPACT) programme (Grant ref. EP/K034510/1).

Appendix A. Supplementary material

References

- [1] S. Casalini, C.A. Bortolotti, F. Leonardi, F. Biscarini, Self-assembled monolayers in organic electronics, *Chem. Soc. Rev.* 46 (2017) 40-71.
- [2] T.J. Huang, B. Brough, C.-M. Ho, Y. Liu, A.H. Flood, P.A. Bonvallet, H.-R. Tseng, J.F. Stoddart, M. Baller, S. Magonov, A nanomechanical device based on linear molecular motors, *Appl. Phys. Lett.* 85 (2004) 5391-5393.
- [3] S.K. Arya, P.R. Solanki, M. Datta, B.D. Malhotra, Recent advances in self-assembled monolayers based biomolecular electronic devices, *Biosens. Bioelectron.* 24 (2009) 2810-2817.
- [4] N.K. Chaki, K. Vijayamohanan, Self-assembled monolayers as a tunable platform for biosensor applications, *Biosens. Bioelectron.* 17 (2002) 1-12.
- [5] D. Samanta, A. Sarkar, Immobilization of bio-macromolecules on self-assembled monolayers: methods and sensor applications, *Chem. Soc. Rev.* 40 (2011) 2567-2592.
- [6] C. Vericat, M.E. Vela, G. Benitez, P. Carro, R.C. Salvarezza, Self-assembled monolayers of thiols and dithiols on gold: new challenges for a well-known system, *Chem. Soc. Rev.* 39 (2010) 1805-1834.
- [7] J.C. Love, L.A. Estroff, J.K. Kriebel, R.G. Nuzzo, G.M. Whitesides, Self-assembled monolayers of thiolates on metals as a form of nanotechnology, *Chem. Rev.* 105 (2005) 1103-1170.
- [8] E. Cortés, A.A. Rubert, G. Benitez, P. Carro, M.E. Vela, R.C. Salvarezza, Enhanced stability of thiolate self-assembled monolayers (SAMs) on nanostructured gold substrates, *Langmuir* 25 (2009) 5661-5666.
- [9] G. Yang, N.A. Amro, Z.B. Starkewolfe, G.-y. Liu, Molecular-level approach to inhibit degradations of alkanethiol self-assembled monolayers in aqueous media, *Langmuir* 20 (2004) 3995-4003.
- [10] E. Delamarche, B. Michel, H. Kang, C. Gerber, Thermal stability of self-assembled monolayers, *Langmuir* 10 (1994) 4103-4108.

- [11] N.T. Flynn, T.N.T. Tran, M.J. Cima, R. Langer, Long-term stability of self-assembled monolayers in biological media, *Langmuir* 19 (2003) 10909-10915.
- [12] J.P. Bearinger, S. Terrettaz, R. Michel, N. Tirelli, H. Vogel, M. Textor, J.A. Hubbell, Chemisorbed poly(propylene sulphide)-based copolymers resist biomolecular interactions, *Nat. Mater.* 2 (2003) 259-264.
- [13] A.K. Nowinski, F. Sun, A.D. White, A.J. Keefe, S. Jiang, Sequence, structure, and function of peptide self-assembled monolayers, *J. Am. Chem. Soc.* 134 (2012) 6000-6005.
- [14] P. Chinwangso, A.C. Jamison, T.R. Lee, Multidentate adsorbates for self-assembled monolayer films, *Accounts Chem. Res.* 44 (2011) 511-519.
- [15] J.B. Schlenoff, M. Li, H. Ly, Stability and self-exchange in alkanethiol monolayers, *J. Am. Chem. Soc.* 117 (1995) 12528-12536.
- [16] J.A. Mann, W.R. Dichtel, Improving the binding characteristics of tripodal compounds on single layer graphene, *ACS Nano* 7 (2013) 7193-7199.
- [17] T. Sakata, S. Maruyama, A. Ueda, H. Otsuka, Y. Miyahara, Stable immobilization of an oligonucleotide probe on a gold substrate using tripodal thiol derivatives, *Langmuir* 23 (2007) 2269-2272.
- [18] Z. Li, R. Jin, C.A. Mirkin, R.L. Letsinger, Multiple thiol-anchor capped DNA-gold nanoparticle conjugates, *Nucleic Acids Res.* 30 (2002) 1558-1562.
- [19] H. Yin, T.J. Deodhar, M. Chen, Y. Lu, J.J. Hu, D. Xiong, Tridentate tripodal sulfur ligand as a stable molecular surface anchor for the fabrication of oligonucleotide-gold based label-free biosensors, *Anal. Methods* 9 (2017) 600-608.
- [20] F. Li, H. Zhang, B. Dever, X.-F. Li, X.C. Le, Thermal stability of DNA functionalized gold nanoparticles, *Bioconjugate Chem.* 24 (2013) 1790-1797.
- [21] A. Fragoso, N. Laboria, D. Latta, C.K. O'Sullivan, Electron permeable self-assembled monolayers of dithiolated aromatic scaffolds on gold for biosensor applications, *Anal. Chem.* 80 (2008) 2556-2563.

- [22] O.Y.F. Henry, J.L.A. Sanchez, C.K. O'Sullivan, Bipodal PEGylated alkanethiol for the enhanced electrochemical detection of genetic markers involved in breast cancer, *Biosens. Bioelectron.* 26 (2010) 1500-1506.
- [23] N. Phares, R.J. White, K.W. Plaxco, Improving the stability and sensing of electrochemical biosensors by employing trithiol-anchoring groups in a six-carbon self-assembled monolayer, *Anal. Chem.* 81 (2009) 1095-1100.
- [24] C. Li, X. Hu, J. Lu, X. Mao, Y. Xiang, Y. Shu, G. Li, Design of DNA nanostructure-based interfacial probes for the electrochemical detection of nucleic acids directly in whole blood, *Chem. Sci.* 9 (2018) 979-984.
- [25] H. Li, Y. Huang, Y. Yu, W. Li, Y. Yin, G. Li, Peptide-based method for detection of metastatic transformation in primary tumors of breast cancer, *Anal. Chem.* 87 (2015) 9251-9256.
- [26] H. Li, Y. Huang, B. Zhang, D. Yang, X. Zhu, G. Li, A new method to assay protease based on amyloid misfolding: application to prostate cancer diagnosis using a panel of proteases biomarkers, *Theranostics* 4 (2014) 701-707.
- [27] H. Li, H. Xie, N. Yang, Y. Huang, L. Sun, G. Li, Design of a bi-functional peptide for protein assays: observation of cortactin expression in human placenta, *Chem. Commun.* 49 (2013) 5387-5389.
- [28] H. Li, H. Xie, Y. Cao, X. Ding, Y. Yin, G. Li, A general way to assay protein by coupling peptide with signal reporter via supermolecule formation, *Anal. Chem.* 85 (2013) 1047-1052.
- [29] M. Frasconi, F. Mazzei, T. Ferri, Protein immobilization at gold–thiol surfaces and potential for biosensing, *Anal. Bioanal. Chem.* 398 (2010) 1545-1564.
- [30] E. González-Fernández, N. Avlonitis, A.F. Murray, A.R. Mount, M. Bradley, Methylene blue not ferrocene: Optimal reporters for electrochemical detection of protease activity, *Biosens. Bioelectron.* 84 (2016) 82-88.
- [31] G. Dekany, L. Bornaghi, J. Papageorgiou, S. Taylor, A novel amino protecting group: DTPM, *Tetrahedron Lett.* 42 (2001) 3129-3132.

- [32] E. González-Fernández, M. Staderini, N. Avlonitis, A.F. Murray, A.R. Mount, M. Bradley, Effect of spacer length on the performance of peptide-based electrochemical biosensors for protease detection, *Sensor Actuat. B-Chem.* 255 (2018) 3040-3046.
- [33] V. Mirčeski, *Square-wave voltammetry theory and application*, Springer, Berlin, 2007.
- [34] R. Campos, E.E. Ferapontova, Electrochemistry of weakly adsorbed species: Voltammetric analysis of electron transfer between gold electrodes and Ru hexamine electrostatically interacting with DNA duplexes, *Electrochim. Acta* 126 (2014) 151-157.
- [35] F. Ricci, N. Zari, F. Caprio, S. Recine, A. Amine, D. Moscone, G. Palleschi, K.W. Plaxco, Surface chemistry effects on the performance of an electrochemical DNA sensor, *Bioelectrochemistry* 76 (2009) 208-213.
- [36] J.-S. Lee, A.K.R. Lytton-Jean, S.J. Hurst, C.A. Mirkin, Silver nanoparticle oligonucleotide conjugates based on dna with triple cyclic disulfide moieties, *Nano Lett.* 7 (2007) 2112-2115.

Author Biographies

Dr. Matteo Staderini

Matteo received his degree in Chemistry and Pharmaceutical Technologies in 2009 from the University of Bologna (Spain). He then moved to Madrid (Spain) where he gained an Interuniversity Masters degree in Medicinal Chemistry (2010) from Complutense, Alcalá and CEU San Pablo Universities. In 2015 he gained his PhD from Complutense University, Madrid (Spain), working on the identification of theranostic small molecules as innovative therapeutic tools. In July 2015 he joined the Prof. Mark Bradley's research group as a Postdoctoral Researcher, where he is currently involved in the IMPACT project working on the development of chemical probes for implantable microsystems for personalised anti-cancer therapy.

Dr. Eva González-Fernández

Eva received her degree in Chemistry in 2007 from the University of Oviedo (Spain) and gained her PhD from the same university in 2012, working on the development of electrochemical nucleic acid-based biosensors. After a period in industry, in January 2014 she joined Prof. Bradley's lab in the University of Edinburgh as a Postdoctoral Researcher, where she is involved in the IMPACT project (EPSRC-funded) working on development of electrochemical biosensors for implantable microsystems for personalised anti-cancer therapy.

Prof. Alan F. Murray

Prof. Murray is Chair of Neural Electronics, assistant principal, academic support and head of the institute for BioEngineering at the University of Edinburgh. He introduced the *Pulse Stream* method for analogue neural VLSI in 1985. Alan's interests are now in (a) direct interaction between silicon and real neuronal cells and (b) silicon chips for biomedical applications. He currently leads the £5.2m IMPACT (Implantable Microsystems for Personalised And Cancer Treatment) project, funded by an EPSRC programme grant. Alan is a fellow of IET, IEEE and the Royal Society of Edinburgh, principal fellow of the HEA and has published over 340 academic papers.

Prof Andrew R Mount

Prof. Mount is Chair of Physical Electrochemistry and Dean of Research in the College of Science & Engineering (CSE) at the University of Edinburgh. His research interests include fundamental and applied electrochemistry, sensing and analysis and the development and application of healthcare and low carbon clean energy technologies.

Prof. Mark Bradley

Prof. Bradley is Chair of Chemical Biology at the School of Chemistry at the University of Edinburgh where he also holds an Honouree Professorship at the Queens Medical Research Institute. His research interests include the application of the tools of chemistry with the

synthesis of materials and molecules to address and solve bio-medical questions and problems. He is Director of the EPSRC funded project Proteus - an Interdisciplinary Research Collaboration in the area of healthcare technologies.

Captions for figures and tables

Scheme 1. Synthesis of TA-1. Reaction conditions: i) DTPM-NMe₂ 1, MeOH, overnight; ii) (3-bromopropoxy)-tert-butyldimethylsilane, NaH, DMF, 24 h; iii) TBAF, THF, overnight; iv) MeSO₂Cl, Et₃N, DCM, 3 h; v) KSAc, anhydrous THF, overnight; vi) HCl/MeOH (1/4), 5 h, reflux; vii) triphenylmethyl chloride, DCM, 3 h; viii) hydrazine monohydrate, 1 h; ix) MB-Phe-Arg-Arg-PEG-2-COOH 10, HOBt, EDC, DMF, 24 h x) TFA/EDT/water/TIS (94/2.5/2.5/1), 30 min.

Figure 1. (A) Representation of the triple and monoanchor probes (TA-1 and MA-1) immobilised onto a gold surface electrode. (B) Percentage MB peak current change with time upon the addition of trypsin for the TA-1 (green circles) and MA-1 surface layers (orange squares). Insets show the calculated fraction of cleavage, θ , vs time plot, and best fit to equation (1) [30]. All points and error bars represent the average and standard deviation respectively of the response from 3 individual sensing layers.

Figure 2. SWV normalised to the corresponding frequency, f , at which they were recorded versus $1/f$ in PBS for (A) MA-sensor and (B) TA-sensor using an amplitude of 25 mV and a step potential of 5 mV.

Figure 3. Percentage MB peak current change with time upon the addition of varying trypsin concentrations (0, 1, 10, 25 and 100 nM) for (A) MA-1 and (B) TA-1 modified surfaces.

Table 1. Analytical characteristics of MA-1 and TA-1 sensors for trypsin detection.

Figure 4. Percentage signal change registered for TA-1 (green circles) and MA-1 (orange squares) probe films when (A) stored in PBS at 4 °C for 30 days; (B) immersed in PBS at 40 °C for 2 h; and (C) immersed in PBS containing 2 mM DTT at room temperature.